



GAP-43 dependency defines distinct effects of netrin-1 on cortical and spinal neurite outgrowth and directional guidance

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ABSTRACT

Growth-associated protein-43 (GAP-43) is a major nervous system protein whose phosphorylation by protein kinase C regulates growth cone responses to extracellular guidance cues via F-actin. GAP-43 is essential for axon pathfinding in both cortical afferents and efferents: when it is genetically deleted, somatosensory, auditory and visual somatotopic maps fail to form, and telencephalic commissural axons fail to cross the midline. Here we investigated whether the midline guidance cue netrin-1 depends on GAP-43 for its functions in neurite growth and guidance. We used 3-dimensional collagen gel co-cultures to show that both endogenous netrin-1, expressed by the spinal cord floor plate, and recombinant netrin-1, expressed by transfected COS7 cells, stimulate neurite outgrowth and chemotropic guidance of neocortical callosal axons. In contrast both were significantly inhibited in GAP-43 (−/−) neocortical callosal axons, mimicking the *in vivo* phenotype. Conversely, neither netrin-1-stimulated neurite outgrowth nor guidance of dorsal spinal cord commissure axons were affected when GAP-43 was absent, again consistent with *in vivo* phenotype but suggesting fundamental differences in how neocortical and spinal cord axons respond to netrin-1. In addition, differences in GAP-43 dependency also distinguished how ventrolateral cortical efferents respond to netrin-1: in contrast to callosal neurites, in which netrin-1 required GAP-43 in order to stimulate both outgrowth and guidance, in ventrolateral efferents, netrin-1 required GAP-43 only to stimulate outgrowth, but not guidance. Moreover, netrin-1 increased the numbers of both types of cortical, but not spinal neurites. The results demonstrate previously unappreciated diversity in how different classes of neurons respond to the same guidance cue.

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1. Introduction

Precisely targeted axon pathfinding is an important characteristic of nervous system development and is achieved by coordination between attractive and repulsive guidance cues and their receptors. Receptors for guidance cues are mainly present on the axonal growth cone, and once activated, trigger cytoplasmic signaling events that eventually lead to reorganization of the growth cone cytoskeleton, which in turn regulates outgrowth and guidance (Guan and Rao, 2003; Lowry and Van Vactor, 2009). The details of precisely how these signaling events cause cytoskeletal reorganization are still far from clear, but actin regulatory proteins

downstream of receptor signaling pathways are usually essential (Lowry and Van Vactor, 2009). For instance, genetic deletion studies have shown that formation of the telencephalic commissures (the anterior and hippocampal commissures and the corpus callosum) that are dependent on the guidance cue netrin-1 (Serafini et al., 1996) and its receptor DCC (Fazeli et al., 1997) also require certain F-actin regulatory proteins such as MARCKs, MacMARCKs, ankyrin, Mena and p190GAP (Brouns et al., 2001; Chen et al., 1996; Lanier and Gertler, 2000; Scotland et al., 1998; Stumpo et al., 1995).

GAP43, is a single copy gene that produces a major growth cone protein, growth-associated protein 43 (GAP-43) whose phosphorylation by PKC in response to extracellular guidance cues, in particular those of the Ig-superfamily, can regulate F-actin behavior in growth cones (Aarts et al., 1998; Nguyen et al., 2009; Walsh et al., 1997). GAP-43 is an essential component of mechanisms regulating cortical axon pathfinding: We have shown that 100% of homozygote GAP-43 (−/−) mice failed to form either somatosensory (cortical barrel) maps or telencephalic commissures (Maier et al., 1999; Shen et al., 2002). GAP-43 is also haploinsufficient for both phenotypes, underscoring its importance in development of these pathways (McIlvain et al., 2003; Shen et al., 2002). The telencephalic commissure phenotype of the GAP-43 knockout mice

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strongly resembles that of the netrin-1 hypomorph and the netrin-1 receptor (DCC) knockout mice (Fazeli et al., 1997; Serafini et al., 1994, 1996). This suggests that GAP-43 (−/−) commissural axons may fail to cross the midline because GAP-43 function is involved in signaling pathways responsive to midline guidance cues such as netrin-1.

Netrin-1 was initially identified as the major attractant that guides spinal commissure axons toward the ventral midline (Kennedy et al., 1994; Serafini et al., 1996). It can also stimulate the outgrowth of spinal commissure neurons *in vitro* (Serafini et al., 1996). The role of netrin-1 in the formation of cortical cytoarchitecture, especially the corpus callosum, is less well understood. The expression of netrin-1 along the tract taken by forebrain commissure neurons and at the point where they cross the midline (Serafini et al., 1996), together with the defects in forebrain commissure formation seen in netrin-1 hypomorph mice indicate that netrin-1 is indispensable for commissure formation in both forebrain and hippocampus (Barallobre et al., 2005; Serafini et al., 1996) and are consistent with a role in either attractive guidance or outgrowth. However the absence of a forebrain midline structure equivalent to the floorplate makes it difficult to differentiate between these two effects on callosal commissure neurons *in vivo*. Moreover, netrin-1 also plays a role in neuronal survival, further complicating interpretation of its function in cortical development (LLambi et al., 2001). Axonal responses to extracellular cues have been measured in two ways: Release of a point source of soluble potential guidance cue by a pipette has been used to measure the actual process of individual growth cone turning (e.g. Ming et al., 1999). A second way, that has the advantage of also allowing neuronal survival/outgrowth responses to be distinguished from directional guidance, co-cultures explants of the neuronal population in question with a cellular source of the putative cue and measures the response of the population of neurites (Guthrie and Lumsden, 1994). Here we have used this latter model to characterize how two different classes of commissural axons – neocortical callosal and spinal commissural, as well as two different classes of neocortical axon, ventrolateral and callosal, respond to recombinant netrin-1 secreted from transfected COS7 cells, or to endogenous netrin-1 expressed by explanted spinal cord floor plate cells. The results demonstrate that netrin-1 has distinct DCC-mediated effects on chemotropic guidance and axon outgrowth in each system. Moreover each DCC-mediated function in each system is differentially dependent on GAP-43, revealing previously unrecognized differences in how netrin-1 elicits its intracellular responses.

2. Materials and methods

2.1. Materials

The pGNET1^{myc} expression plasmid that contains the coding region of the chick netrin-1 cDNA and a myc tag was kindly provided by Dr. Mark Tessier-Lavigne. Control vector was prepared by excision of the netrin-1 cDNA sequence. Monoclonal antibodies were DCC (clone AF5, Oncogene); Myc (clone 9E10, Sigma); Vimentin (clone V9, Santa Cruz Biotechnology) and TAG-1 (4D7, Hybridoma Bank). Cell culture used Vitrogen 100 (Cohesion Technologies). Media components and LipofectAMINE for transient transfections were from GIBCO.

2.2. Immunocytochemistry

Tuj-1, DCC and TAG-1 immunostaining was done on cultured explants. For Tuj-1 and TAG-1 double staining, explants in collagen gel were first fixed in 4% paraformaldehyde (PFA) and pretreated with 0.1 M NaOH for 1 h. After neutralization with 0.1 M sodium acetate (pH 5.5) explants were blocked with 10% goat serum in PBS and permeabilized with 0.1% digitonin for 4 h. For TAG-1 and DCC immunostaining, explants in laminin coated culture dishes were permeabilized with 0.01% digitonin and incubated overnight with the primary antibodies. In each case antibody immunoreactivity was visualized with either FITC or Texas-red conjugated secondary antibodies. Tuj-1 and TAG-1 double labeled images were collected with a SPOT digital camera mounted on a Nikon Eclipse E800 microscope. TAG-1 and DCC

double-labeled images were collected sequentially, using a Leica TCS SP2 confocal microscope at settings optimized for maximal resolution.

2.3. Transient transfections

COS7 cells were cultured in DMEM + 10% FBS at 37 °C in a humidified 5% CO₂ incubator. Cells at 70–80% confluence were transfected either with pGNET1^{myc} or control plasmid using LipofectAMINE, a day prior to use in co-culture experiments. Efficiency of transfection was monitored by immunostaining with 9E10 anti-myc monoclonal antibody. Vector transfected cells were used as controls.

2.4. Collagen gel explant co-cultures

Timed pregnant GAP-43 (+/−) mice (noon of the day on which plug is found is E0.5) were used to collect E12.5 or E15.5 embryos. For the E12.5 cultures, all of the neocortex was used to prepare ventrolateral neocortical efferents. For the E15.5 cultures, the area of the neocortex previously described as giving rise to callosal axons was separated from the rest of the cortex (Ozaki and Wahlsten, 1998). After dissection and removal of the pial membranes, tissue was cut into pieces about 200 μm × 200 μm with a McIlwain tissue chopper. Floor plate and dorsal spinal cord tissues were dissected from E12.5 embryos after opening the spinal cord at the roof plate in an open book configuration. Tissue pieces were kept in cold L-15 medium supplemented with 0.6% glucose until plating. A small piece of tissue was collected from each embryo for PCR-genotyping, as described previously (Maier et al., 1999). Netrin-1 or control vector-transfected COS7 cells were collected by centrifuging mechanically detached cells, and cell pellets were mixed with collagen gel (900 μl of Vitrogen100; 100 μl of 10× DMEM and 23 μl of 1 M NaHCO₃) at approximately 500 cells/μl. A 5–6 μl spot of cell-gel mixture was dotted onto the bottom of 8-well Labtek chamber slides and allowed to gel in a 37 °C incubator for 20 min. Next, the gel-encapsulated cells were overlaid with 100 μl of collagen gel, and both +/+ and −/− tissue pieces were placed in the same well, 150–250 μm on either side of the gel-encapsulated cells. After the collagen gelled, 500 μl of culture medium (DMEM with GlutaMAX, 10% horse serum and 0.1% penicillin-streptomycin) was added to each well. Explants were cultured for 24–48 h in a humidified 5% CO₂ incubator at 37 °C.

2.5. Antibody blocking experiments

Anti-DCC monoclonal antibody (clone AF5, IgG1, Oncogene) was added to both the collagen gel and the culture medium at concentrations of either 2 μg/ml or 5 μg/ml. As control, IgG concentration and subtype matched anti-vimentin monoclonal antibody (V9, IgG1, Santa Cruz Biotechnology) was added to the gel and medium. To prevent endocytosis of any antigen–antibody complex, 0.005% sodium azide was added along with antibodies (Meiri et al., 1988).

2.6. Imaging and quantification of neurite outgrowth

DIC images of explants at 24 h after plating were collected using a Spot camera mounted on a Nikon Eclipse TE200 inverted microscope. Neurites were counted and measured using IP lab software (Scanalytics). Initially, all neurites in every focal plane were counted, but it was found that the results correlated well with measurements from the focal plane having the majority of the neurites. Thus results presented here derive from measurements made on the focal plane selected for having the maximum number of neurites. To measure chemotropic guidance of neurites toward the netrin-1-expressing COS7 cells, the image of the explant was bisected using the outline of the cell-gel capsule as a template. A response was defined as attractive when >70% of the total neurites were found in the hemisphere adjacent to the cell/gel capsule, whereas a response was defined as repulsive when >70% of the total neurites grew in the hemisphere distant from the cell/gel capsule. A response was defined as neutral when neurites in neither hemisphere reached >70% of total. Data were analyzed using Microsoft Excel and the mean and s.e.m. are presented. The number of neurites in the proximate half of the explants was counted using IPLab, focusing on the neurite tip rather than at the region where the neurite emerges from the explant. Statistical significance used either ANOVA analysis of variance and Student's *t*-test or χ^2 tests comparing (−/−) explants with expected values based on (+/+) responses in the presence of netrin-1.

3. Results

3.1. Netrin-1-mediated chemotropic guidance and outgrowth of neocortical (callosal) neurites requires functional DCC

We used tissue pieces dissected from the area of neocortex previously described as giving rise to callosal axons in E15.5–E16.5 mouse brain (Ozaki and Wahlsten, 1998) in collagen gel co-culture experiments with COS7 cells engineered to express netrin-1. Tuj-1/TAG-1 immunolabeling confirmed that the majority of the

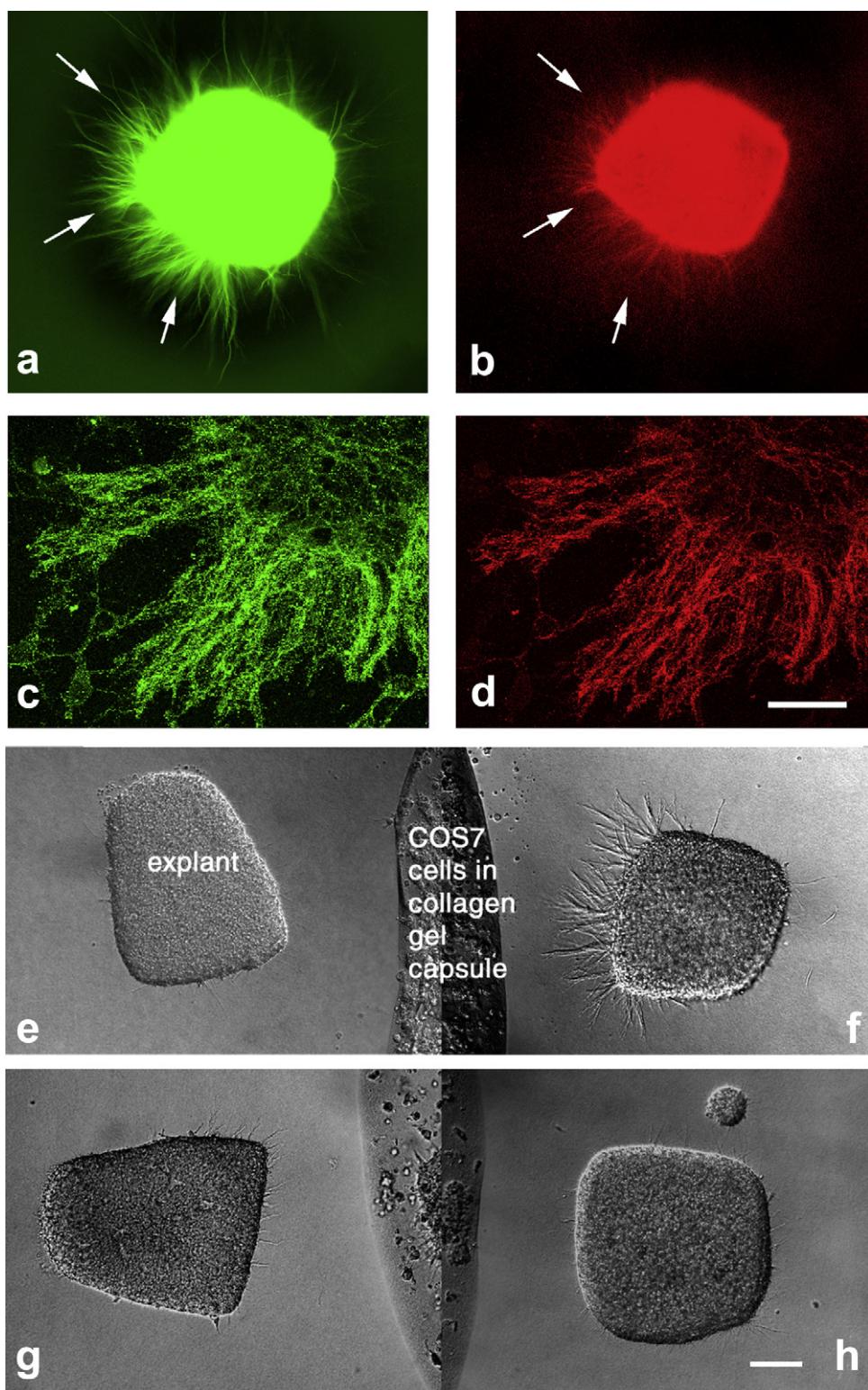


Fig. 1. DCC is expressed by callosal neurites, and required for their chemotropic guidance response to netrin-1. (a and b) E15.5 neocortical explants in collagen gels immunostained with Tuj-1 (a) and the callosal marker TAG-1 (b), followed by FITC and Texas-red conjugated secondary antibodies respectively. TAG-1 labeled axons are arrowed. (c and d) Similar neocortical explants co-immunostained with TAG-1 (c) and DCC (d). TAG-1 positive neurites also expressed DCC, indicating their responsiveness to netrin-1. Scale bar: 100 μm for (a) and (b); 40 μm for (c) and (d). (e–h) Representative DIC images of E15.5 GAP-43 (+/+) explants from the area of the neocortex that gives rise to TAG-1/DCC labeled callosal neurons after 24 h suspended in a collagen gel. (e) Explant co-cultured with COS7 cells transfected with pGNET1^{myc} control vector. (f) Explant co-cultured with COS7 cells transfected with the pGNET1^{myc}/netrin-1 construct in the presence of 2 μg/ml control antibody (anti-vimentin), showing the attractive response of the explants, stimulation of neurite outgrowth and increased numbers of netrin-1 responsive neurons compared with controls. (g) Explants cultured with COS7 cells transfected with the pGNET1^{myc}/netrin-1 construct in the presence of 2 μg/ml anti-DCC antibody. (h) Explant cultured with COS7 cells transfected with the pGNET1^{myc}/netrin-1 construct in the presence of 5 μg/ml anti-DCC antibody (scale bar = 40 μm).

neurites extended by these explants were labeled with the callosal marker TAG-1 (Wolfer et al., 1994) while TAG-1/DCC immunolabeling confirmed that the TAG-1 labeled neurites also expressed the netrin-1 receptor DCC (Fig. 1a–d). Netrin-1 was first reported as an axon guidance cue required for both spinal cord and telencephalic commissure formation (Serafini et al., 1996). Its function in chemotropic guidance has been quantified in co-culture experiments by determining the sidedness of neurite outgrowth toward a cellular source of netrin-1 (Guthrie and Lumsden, 1994; Kennedy et al., 1994). Fig. 1e–h demonstrates that netrin-1 mediates chemotropic guidance in these co-cultures using the criteria established by Guthrie and Lumsden (1994). Section 2 provides details of how this chemotropic guidance was quantitated.

Fig. 2a shows the results of this quantitation. When GAP-43 (+/+) callosal explants were positioned <300 μ m away from a COS7 cell/gel capsule expressing vector alone, only 3% of the explants extended >70% of their total neurites in the half of the explants proximal to the transfected cells, indicating an attractive response. In contrast when they were positioned adjacent to a COS7 cell/gel capsule expressing netrin-1, >90% of the explants displayed an attractive response to the netrin-1 expressing cells. Addition of anti-DCC antibody to both collagen gel and media caused a dose-dependent inhibition of the netrin-1-mediated attraction: Thus, 2 μ g/ml anti-DCC antibody significantly inhibited the attractive response while 5 μ g/ml anti-DCC antibody reduced it to control levels. Hence, netrin-1 mediated chemotropic guidance of callosal axons is dependent on the netrin-1 receptor, DCC.

We also compared the length of neurites growing toward cells expressing vector alone with those growing toward cells expressing netrin-1. After 24 h in co-culture the average length of neurites growing toward COS7 cells expressing netrin-1 significantly increased compared with neurites growing toward COS7 cells transfected with vector alone ($p < 0.001$, ANOVA and 2-tailed *t*-test). Hence, netrin-1 has similar neurite outgrowth-promoting activity for callosal axons, as has been reported for dorsal spinal cord commissure neurons (Serafini et al., 1994). To verify that this neurite outgrowth-promoting activity is also mediated through DCC, as it is in the spinal cord (Fazeli et al., 1997), we repeated the netrin-1 experiment in the presence of either 2 or 5 μ g/ml DCC antibody in both the culture medium and in the collagen gel. Presence of both 2 and 5 μ g/ml anti-DCC antibody significantly inhibited the neurite outgrowth-promoting activity of netrin-1 ($p < 0.001$, ANOVA and 2-tailed *t*-test). Thus, netrin-1 effects on callosal neurite outgrowth mimic those in the spinal cord and are also mediated by DCC.

3.2. Netrin-1 stimulation of E15.5 neocortical (callosal) neurite chemotropic guidance and outgrowth is significantly inhibited when GAP-43 is absent

Genetic deletion of both alleles of the GAP-43 gene completely prevents formation of the corpus callosum *in vivo*, whereas deletion of one allele is sufficient to inhibit callosal formation (Shen et al., 2002). These defects are not related to failure of the midline to fuse (Shen et al., 2002), and neither is the midline of the DCC null mouse defective (Fazeli et al., 1997), suggesting that any deficits are autonomous to the callosal axons themselves. To determine whether the GAP-43 defects are mediated *via* netrin-1, we co-cultured explants from GAP-43 (+/+) (+/–) and (–/–) mice with transfected COS7 cells and measured chemotropic guidance and neurite outgrowth as before. Fig. 3a shows that neither GAP-43 (+/+) nor (–/–) explants (not shown) displayed attractive responses to COS7 cells transfected with control vector, whereas 95% of GAP-43 (+/+) explants were attracted by COS7 cells expressing netrin-1. In contrast only 35.4% of GAP-43 (–/–) explants displayed attractive responses, while the remainder displayed either neutral

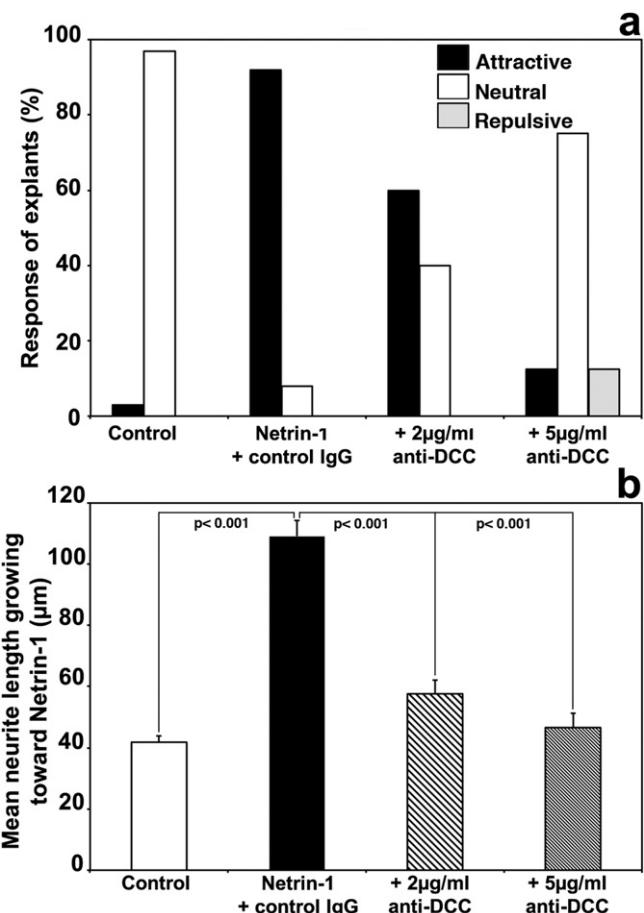


Fig. 2. Netrin-1 stimulated guidance and outgrowth are dependent on the DCC receptor. (a) The response of each E15.5 neocortical explant after 24 h in co-culture with COS-7 cells was rated as attractive (black bars), neutral (white bars) or repulsive (gray bars) according to the distribution of neurites between the proximate and distant halves of the explant (see Section 2). In the presence of COS7 cells expressing vector alone with control (anti-vimentin IgG) in the media, 97% of explants responded neutrally whereas in the presence of COS7 cells expressing netrin-1 and control antibody (anti-vimentin IgG) in the media, 92.5% of the explants (12/13) were attracted. When 2 μ g/ml anti-DCC was added, the number of explants displaying attraction was reduced to 60% (6/10), whereas in the presence of 5 μ g/ml of anti-DCC the number was further reduced to 12.5% (1/8). The reduction at 5 μ g/ml was significantly different ($p < 0.0001$, $\chi^2 > 305$ compared with the response to netrin-1). (b) The mean length of 100 E15.5 callosal neurites from each explant was calculated. (White bar) Neurons co-cultured with explants expressing vector alone in the presence of control (anti-vimentin IgG). Mean length was $41.82 \pm 1.98 \mu\text{m}$ (s.e.m.) $n = 10$ explants. (Black bar) Neurons co-cultured with explants expressing netrin-1 in the presence of control (anti-vimentin IgG). Mean length was $109 \pm 5.1 \mu\text{m}$ (s.e.m.) $n = 10$ explants. $p < 0.001$ compared with control, 2-tailed *t*-test. (Diagonal bar) Neurons co-cultured with explants expressing netrin-1 in the presence of 2 μ g/ml anti-DCC antibody. Mean length was $57.6 \pm 13.1 \mu\text{m}$ (s.e.m.), $n = 8$ explants. $p < 0.001$ compared with netrin-1, 2-tailed *t*-test. (Gray bar) Neurons co-cultured with explants expressing netrin-1 in the presence of 5 μ g/ml anti-DCC. Mean length was $46.6 \pm 13.1 \mu\text{m}$ (s.e.m.), $n = 8$ explants. $p < 0.001$ compared with netrin-1, 2-tailed *t*-test.

or repulsive responses, indicating that absence of GAP-43 can significantly inhibit netrin-1 mediated chemotropic guidance. Interestingly, the number of GAP-43 heterozygote (+/–) explants displaying attractive responses toward netrin-1 was also reduced. These results indicate netrin-1-mediated chemotropic guidance of callosal neurons is dependent on GAP-43, and also suggest that disrupted netrin-1 signaling may contribute to the haploinsufficiency of GAP-43 (+/–) in callosal formation *in vivo* (Shen et al., 2002).

With respect to whether GAP-43 affects the netrin-1 stimulation of neurite outgrowth, the mean length of E15.5 neocortical callosal neurites from both GAP-43 (+/+) and GAP-43 (–/–) neurons

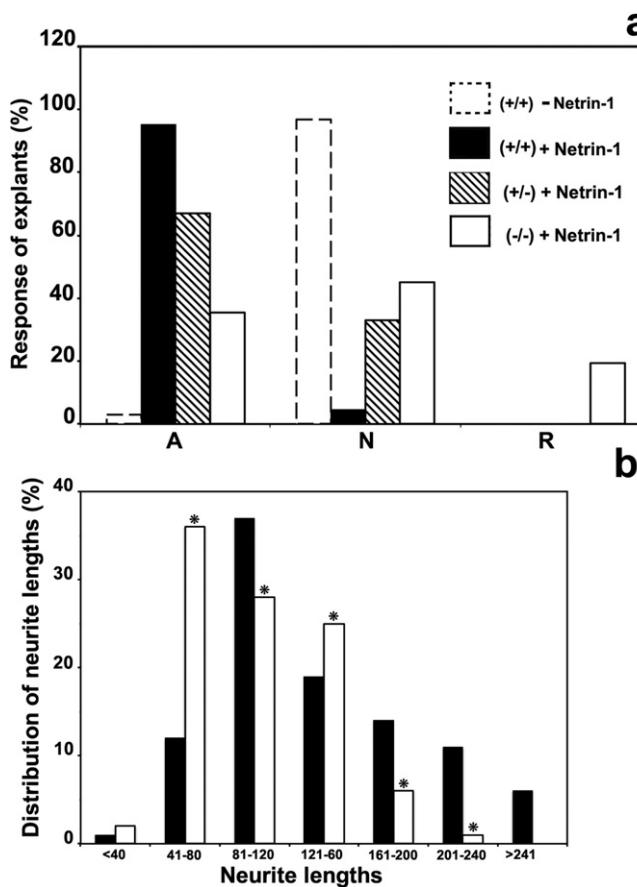


Fig. 3. Netrin-1 stimulated guidance and outgrowth of E15.5 neocortical callosal neurons is dependent on GAP-43. (a) The response of each E15.5 neocortical explant after 24 h in co-culture was rated as attractive (A), neutral (N) or repulsive (R) response according to the distribution of neurites between the proximate and distant halves of the culture as before. (Dashed bar) GAP-43 (+/+) explants co-cultured in the presence of vector transfected COS7 cells. 97.5% (39/40) of explants displayed a neutral response. (Black bar) GAP-43 (+/+) explants co-cultured in the presence of netrin-1 transfected COS7 cells. 95.3% (61/64) of GAP-43 (+/+) explants displayed an attractive response; the remainder displayed a neutral response. (Diagonals) GAP-43 (+/-) explants co-cultured in the presence of netrin-1 transfected COS7 cells. 67% (16/24) of explants displayed an attractive response and the remainder had a neutral response. $p < 0.001$, $\chi^2 > 35$ compared with (+/+) response to netrin-1. (White bar) GAP-43 (-/-) explants co-cultured in the presence of netrin-1 transfected COS7 cells. Only 35.4% or 29/82 displayed an attractive response. The remainder either displayed a neutral response (45% or 37/82) or a repulsive response (19.5% or 16/82). $p < 0.001$, $\chi^2 > 305$ compared with (+/+) response to netrin-1. (b) The length of neurites elaborated in the proximal half of each explant in response to netrin-1, ranked according to size. (Black bars) GAP-43 (+/+) neurites, $n = 648$ from 13 explants. Most neurites were between 81 and 120 μm , a 2–3-fold induction in length. 35% of all neurites were greater than 160 μm , a 4-fold induction in length. (White bars) GAP-43 (-/-) neurites, $n = 534$ from 10 explants. Most neurites were between 40 and 80 μm a 2-fold induction in length and 7% of all neurites were greater than 160 μm , a 4-fold induction in length. * $p < 0.001$ compared with wild type (ANOVA and 2-tailed *t*-test).

growing toward COS7 cells expressing vector alone was similar ($44.06 \pm 3.33 \mu\text{m}$ (s.e.m.), $n = 100$ neurites from 5 explants, and 39.3 ± 2.98 (s.e.m.), $n = 100$ neurites from 5 explants respectively). In the presence of netrin-1 the mean length of both GAP-43 (+/+) and (-/-) neurites increased significantly to $113 \pm 1.9 \mu\text{m}$ (s.e.m.), $n = 648$ neurites from 13 explants, and $80 \pm 1.7 \mu\text{m}$ (s.e.m.) $n = 424$ neurites from 13 explants respectively. Both increases were significantly different from controls ($p < 0.001$, ANOVA followed by Student's *t*-test) and but the response of GAP-43 (-/-) neurites was significantly less than GAP-43 (+/+) ($p < 0.05$ ANOVA and 2-tailed *t*-test). Further investigation demonstrated the cause of the difference in how netrin-1 stimulated outgrowth in the two populations: Whereas netrin-1 could stimulate a 4 fold or greater

increase in length from (40 to $>160 \mu\text{m}$) in 26% of GAP-43 (+/+) neurites, it could only stimulate a similar degree of outgrowth in 7% of GAP-43 (-/-) neurites. Hence while GAP-43 is not required for baseline (presumably integrin-mediated) outgrowth of neocortical callosal neurites, as has been shown previously in the PNS (Strittmatter et al., 1995) and in cerebellar granule cells (Meiri et al., 1998), absence of GAP-43 significantly inhibits the ability of netrin-1 to stimulate maximal outgrowth of a subset of callosal neurons. Thus GAP-43 is required for both netrin-1 mediated chemotropic guidance and netrin-1 mediated stimulation of maximal callosal neurite outgrowth.

3.3. GAP-43 is not required for spinal commissure formation, and netrin-1 stimulation of spinal commissure neurite chemotropic guidance and outgrowth is not affected when GAP-43 is absent

Netrin-1 was first described as a guidance cue for midline crossing via the spinal commissure system, and neither the netrin-1 hypomorph nor DCC knockout mice generate spinal commissures (Fazeli et al., 1997; Serafini et al., 1996). Nonetheless even though the corpus callosum is absent in 100% of GAP-43 (-/-) mice *in vivo*, the spinal commissure appears normal (Fig. 4a). To investigate the role of GAP-43 in netrin-1 effects on spinal commissure neurons, we generated dorsal spinal explants from E12.5 mouse embryos and co-cultured them either with netrin-1 transfected COS7 cells as before, or with E12.5 floorplate tissue as a source of endogenous netrin-1 (Fig. 4c). Fig. 4d shows that, as with the neocortical (callosal) explants, most of the GAP-43 (+/+) spinal cord explants displayed a neutral response to COS7 cells expressing vector alone, but when the COS7 cells expressed netrin-1, 100% displayed an attractive response. However, and in striking contrast to the neocortical (callosal) explants, most (82% or 9/11) of the GAP-43 (-/-) spinal commissure neuron explants also displayed an attractive response to the netrin-1 expressing COS7 cells, while the remainder had a neutral response. The results were replicated when floorplate was used as an endogenous source of netrin-1. Fig. 4e shows both GAP-43 (+/+) and (-/-) neurons displaying an attractive response to the floorplate explants. In this case fewer total GAP-43 (+/+) explants displayed an attractive response to the floorplate cultures (80% or 8/10) than COS7 cells, probably reflecting the narrower effective range of netrin-1 secreted by the floorplate: floorplate was unable to attract the neurons if it was more than 300 μm away from dorsal spinal cord explants, whereas COS7 cells were effective over more than 400 μm . The differences between the spinal cord and callosal neurites to netrin-1 were not limited to chemotropic guidance: netrin-1 significantly increased the mean length of both GAP-43 (+/+) and (-/-) spinal commissure neurites by similar amounts as callosal neurites ($145 \pm 6.2 \mu\text{m}$ (s.e.m.), $n = 148$ neurites from 5 explants, and $131 \pm 5.1 \mu\text{m}$ (s.e.m.), $n = 104$ neurites from 5 explants respectively). Similarly netrin-1 could stimulate maximal increase in length in a similar proportion of GAP-43 (+/+) spinal commissure neurites as GAP-43 (+/+) callosal neurites (35.8% vs. 26%). However in contrast with callosal neurites netrin-1 could also stimulate a maximal increase in length of 30% of GAP-43 (-/-) spinal commissure neurites, up from 7% in the GAP-43 (-/-) callosal explants. Together the results demonstrate that netrin-1 does not require GAP-43 in order to stimulate either chemotropic guidance or maximal outgrowth of dorsal spinal commissure neurons.

3.4. In ventrolateral neocortical efferents, GAP-43 is not required for chemotropic guidance but is required for neurite outgrowth

The foregoing results suggest that netrin-1-mediated chemotropic guidance and neurite outgrowth are regulated differently in different populations of neurons that cross the midline. To investigate the extent of these differences further, we

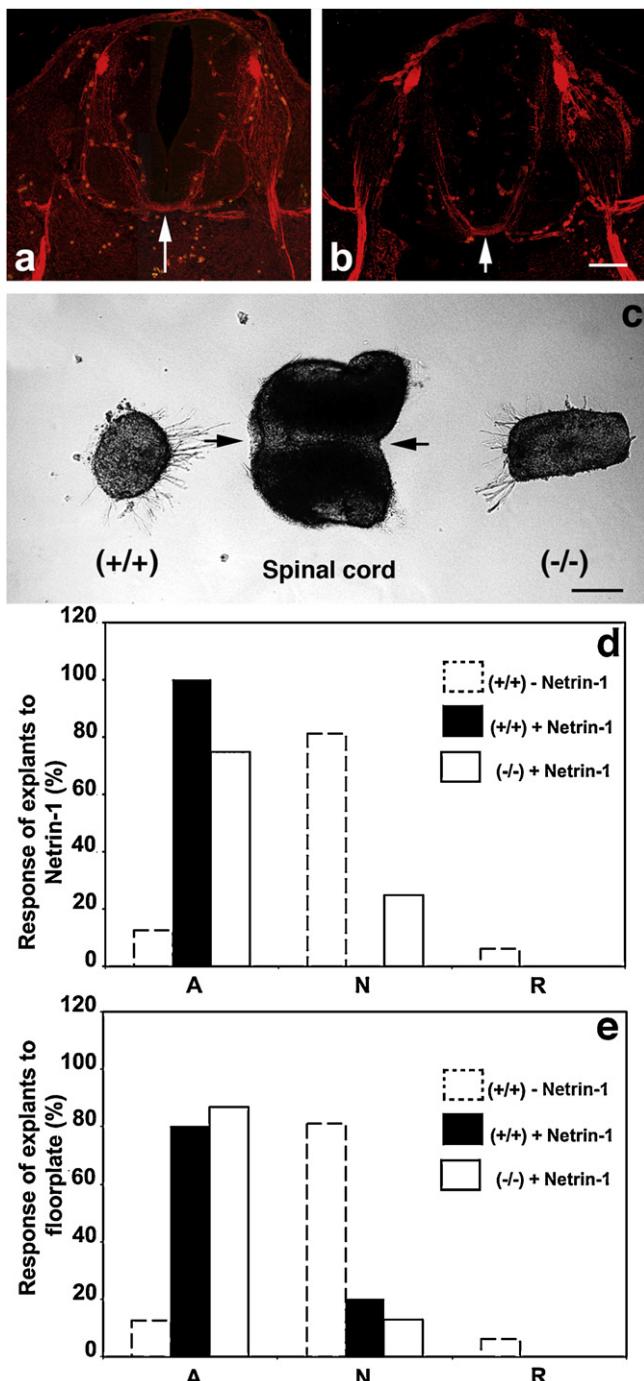


Fig. 4. Netrin-1 stimulated chemotropic guidance of E12.5 spinal cord commissure neurons to netrin-1 is independent of GAP-43. (a and b) Frozen sections of P1 spinal cord labeled with TAG-1 antibody followed by Texas Red-conjugated secondary antibodies to reveal the spinal commissure (arrows). (a) GAP-43 (+/+) and (b) GAP-43 (-/-) demonstrating the spinal commissure (arrowed). Scale bar is 100 μ m. (c) Representative DIC image of E12.5 GAP-43 (+/+) and (-/-) dorsal spinal neuron explants co-cultured for 24 h with E12.5 spinal cord, floorplate region indicated with arrows. Scale bar is 30 μ m. (d and e) E12.5 dorsal spinal neurons co-cultured for 24 h with either COS7 cells or spinal cord as a source of netrin-1. (d) Co-cultured with COS7 cells. The response of each explant was rated as attractive (A), neutral (N) or repulsive (R). (Dashed bar) GAP-43 (+/+) neurons cultured in the presence of COS7 cells expressing vector. 87% (13/15) of explants had a neutral response. (Black bar) GAP-43 (+/+) neurons cultured in the presence of COS7 cells expressing netrin-1. 100% (8/8) displayed an attractive response. (White bar) GAP-43 (-/-) neurons cultured in the presence of COS7 cells expressing netrin-1. 82% (9/11) of dorsal spinal explants displayed an attractive response while 18% (2/11) had a neutral response. The difference between (+/+) and (-/-) were not significant (χ^2). (e) Co-cultured with E12.5 spinal cord. (Dashed bar) GAP-43 (+/+) neurons cultured in the presence of COS7 cells expressing vector as above. (Black bar) GAP-43 (+/+) neurons cultured in the presence of E12.5 spinal cord. 80% (8/10) displayed an attractive response whereas 20% (2/10) had a neutral response. (White bar) GAP-43 (-/-) neurons cultured in the presence of E12.5 floorplate explants. 87% (20/23) displayed an attractive response whereas 13% (3/23) had a neutral response. The difference between (+/+) and (-/-) were not significant (χ^2).

turned to another netrin-1-dependent system in cortex: cortical efferents follow two main pathways: the telencephalic commissures we already investigated comprise one. The second efferent pathway descends ventrolaterally through the internal capsule in the ventral forebrain to the ipsilateral thalamus, midbrain and spinal cord. The cortical efferents responsible for both pathways are both born around E12 (Koester and O'Leary, 1993, 1994) however there is a substantial temporal disparity in when they send out axons: the first ventrolateral axons are elaborated and enter the internal capsule at E12–13 (De Carlos and O'Leary, 1992), whereas the first callosal axons are only elaborated at E15 (Koester and O'Leary, 1994). Like the callosal axons, the early ventrolateral axons are also responsive to netrin-1 *in vivo* (Richards et al., 1997). We therefore co-cultured neocortical explants from E12.5 mice with netrin-1 transfected COS7 cells or floorplate as before, and measured chemotropic guidance and outgrowth (Fig. 5a). Fig. 5b and c shows that ventrolateral cortical efferents display guidance responses to netrin-1 whether GAP-43 was present or not, and whether COS7 cells (Fig. 5b) or floorplate (Fig. 5c) was used as a source of netrin-1. With respect to neurite outgrowth, netrin-1 was less effective at stimulating outgrowth of ventrolateral cortical axons compared with either callosal or spinal commissural axons. Thus the mean length of the GAP-43 (+/+) and (-/-) neurites attracted by netrin-1 was 66.34 μ m \pm 2.34 (s.e.m.), n = 100 neurites from 3 explants, and 50.23 μ m \pm 1.71 (s.e.m.), n = 100 neurites from 3 explants respectively. The differences in mean length was between (+/+) and (-/-) neurites was significant (p < 0.05, paired *t*-test) indicating that netrin-1's reduced ability to stimulate neurite outgrowth in these neocortical efferents also involves GAP-43. Likewise netrin-1 stimulated similar much smaller maximal increases in length (1.8 fold and 1.5 fold respectively) in similar proportions of GAP-43 (+/+) and (-/-) ventrolateral neurites (8%). These results suggest that the populations of neurites present in the callosal explants that are maximally responsive to netrin-1 and particularly dependent on GAP-43 are absent from ventrolateral cortical efferents.

3.5. Netrin-1-stimulated increases in neurite numbers are GAP-43 dependent in cortical efferent explants, but not spinal commissure explants

We noticed that co-culturing neurons with a source on netrin-1 increased not only the length but also the number of neurites in the proximate hemisphere of the explant, and therefore we also quantitated this response in each of the 3 systems we had investigated. Although visual inspection suggested that there were no significant differences in fasciculation between GAP-43 (+/–) and (–/–) neurites, we attempted to mitigate the effects of fasciculation by counting the tips of neurites rather the region where the neurites emerge from the explant and where fasciculation is expected to be most pronounced. In the absence of netrin-1 the mean number of neurites per proximate hemisphere of the neocortical callosal explants was similar whether or not GAP-43 was present (22.4 ± 2.87 (s.e.m.), n = 10 explants and 29.6 ± 6.5 (s.e.m.), n = 5 explants respectively). When GAP-43 (+/+) explants from either E12.5 ventrolateral cortex or E15.5 callosal cortex were cultured in the presence of netrin-1 expressing COS7 cells this number increased more than 4 fold to 96.42 ± 9.57 (s.e.m.), n = 10 explants and 88.46 ± 3.63 (s.e.m.), n = 10 explants respectively (p < 0.001, ANOVA followed

cells expressing vector as above. (Black bar) GAP-43 (+/+) neurons cultured in the presence of E12.5 spinal cord. 80% (8/10) displayed an attractive response whereas 20% (2/10) had a neutral response. (White bar) GAP-43 (–/–) neurons cultured in the presence of E12.5 floorplate explants. 87% (20/23) displayed an attractive response whereas 13% (3/23) had a neutral response. The difference between (+/+) and (–/–) were not significant (χ^2).

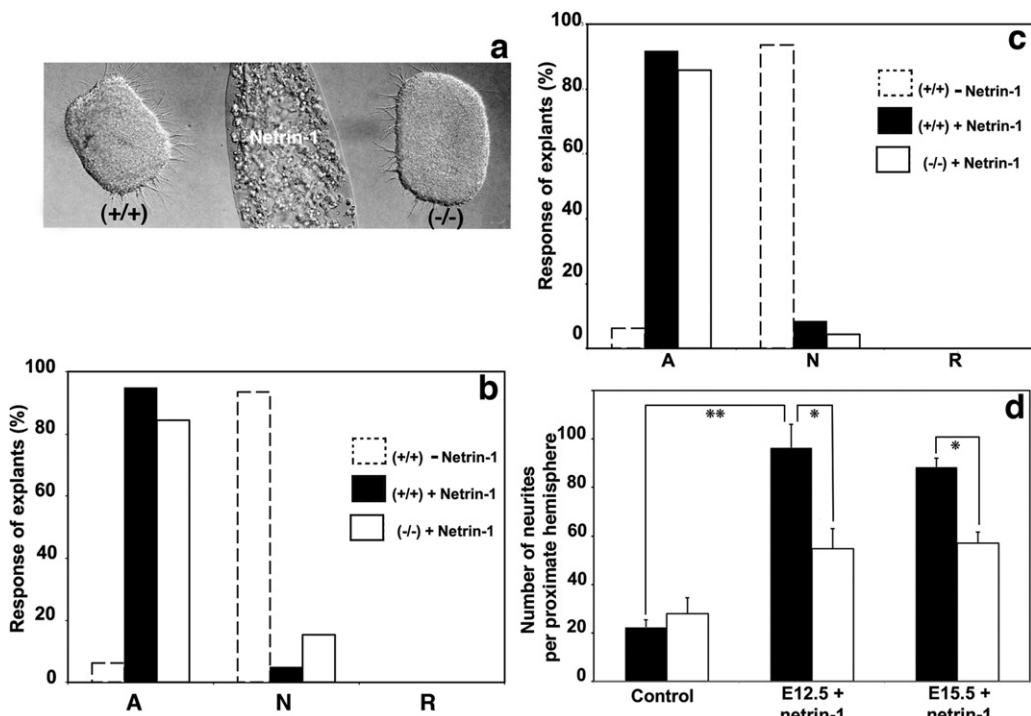


Fig. 5. Netrin-1-stimulated chemotropic guidance of E12.5 neocortical explants is GAP-43 independent, whereas netrin-1-stimulated neurite outgrowth and numbers of neurites both require GAP-43. (a) Representative DIC image of E12.5 GAP-43 (+/+) and (−/−) neocortical explants co-cultured for 24 h co-cultured with transfected COS7 cells. (b) The response of each explant was rated as attractive was rated as attractive (A), neutral (N) or repulsive (R) response according to the distribution of neurites between the proximal and distant halves of the culture as before. (Dashed bar) GAP-43 (+/+) explants co-cultured in the presence of vector transfected COS7 cells 94% (15/16) of explants had a neutral response. (Black bar) GAP-43 (+/+) neurons co-cultured in the presence of netrin-1 transfected COS7 cells. 95% (19/20) were attracted, 5% (1/20) had a neutral response. (White bar) GAP-43 (−/−) neurons co-cultured in the presence of netrin-1 transfected COS7 cells. 85% (22/26) were attracted, 15% (4/26) had a neutral response. The differences between (−/−) and (+/+) in the presence of netrin-1 were not significant (χ^2). (c) E12.5 neocortical explants co-cultured for 24 h with E12.5 floorplate explants as a source of netrin-1. The response of each explant was rated as attractive was rated as attractive (A), neutral (N) or repulsive (R) response according to the distribution of neurites between the proximal and distant halves of the culture as before. (Dashed bars) GAP-43 (+/+) neurons co-cultured in the presence of vector transfected COS7 cells 94% (15/16) of explants had a neutral response. (White bars) GAP-43 (−/−) neurons cultured in the presence of floorplate. 86% (12/14) were attracted, 14% (2/14) had a neutral response. The differences between (−/−) and (+/+) in the presence of netrin-1 were not significant (χ^2). (d) The mean number of neurites in the half of the explant proximate to the COS7 cells in each of the cortical explants investigated. (Black bars) GAP-43 (+/+/), (white bars) GAP-43 (−/−). (Control) The mean number of E12.5 neocortical ventrolateral neurites in the proximate half of each explant in response to COS7 cells expressing vector. (E12.5 + netrin-1) The mean number of E12.5 neocortical ventrolateral neurites in the proximate half of each explant in response to COS7 cells expressing netrin-1. (E15.5 + netrin-1) The mean number of E15.5 neocortical callosal neurites in the proximate half of each explant in response to COS7 cells expressing netrin-1. (**) Indicates significant differences ($p < 0.001$) between (+/+) controls and netrin-1 treatment ($p < 0.001$, two tailed t -test); (*) indicates significant differences between (+/+) and (−/−) plus netrin-1 ($p < 0.001$, two tailed t -test).

by Student's *t*-test compared with controls). In the absence of GAP-43 the ability of netrin-1 to increase the number of neurites in both callosal and ventrolateral explants was significantly reduced by similar amounts 54.82 ± 8.26 (s.e.m.), $n = 8$ explants and 56.91 ± 4.63 (s.e.m.), $n = 8$ explants) respectively $p < 0.001$ for each compared with control (ANOVA followed by Student's *t*-test compared with controls). In striking contrast netrin-1 did not increase the numbers of spinal commissure neurites whether or not GAP-43 was present (20.8 ± 4.3 (s.e.m.), $n = 5$ explants and 28 ± 6.6 (s.e.m.), $n = 10$ explants) respectively. The results indicate another difference in the response of spinal *versus* cortical neurons to netrin-1.

4. Discussion

Here we have used *in vitro* 3D co-culture models that have previously been used to measure the chemotropic guidance, outgrowth and survival responses of populations of neurites to putative extracellular cues to demonstrate three novel findings about how netrin-1 regulates growing neurites: First we have shown that there are significant differences in the way netrin-1 affects cortical vs. spinal commissure neurons: in cortical commissure neurons netrin-1 stimulates chemotropic guidance and outgrowth and increases the numbers of netrin-1 responsive neurons, whereas in spinal commissure neurons netrin-1 has no effect on the numbers of responsive neurons. Second we have

shown that there are significant distinctions in the way different commissural neurons respond to netrin-1: whereas the effects of netrin-1 on chemotropic guidance, outgrowth and number of neocortical callosal axons all require GAP-43, effects of netrin-1 on chemotropic guidance and outgrowth of spinal commissure neurons are totally GAP-43 independent, even though both classes of neurons required a functional DCC receptor to achieve their netrin-1 responses. Finally we have shown that in another class of netrin-1-responsive cortical efferents, that are ventrolaterally rather than commissurally directed, GAP-43 dependency can decouple the different netrin-1 responses: whereas netrin-1 does not require GAP-43 to stimulate either chemotropic guidance or maximal outgrowth of these neurites, it is dependent on GAP-43 to stimulate increased numbers of responsive neurons. This careful and rigorous dissection of netrin-1-mediated effects now sets the stage for a thorough analysis of the underlying molecular mechanisms that give rise to these distinctions.

4.1. Netrin-1 has distinct effects on commissural axons

Netrin-1 is considered to be the major midline guidance molecule across the spinal commissure in vertebrates (Chiscolm and Tessier-Lavigne, 1999). Its ability to stimulate both guidance and outgrowth of GAP-43 (+/+) mouse spinal cord commissure neuron explants *in vitro* replicated previous reports (Kennedy

et al., 1994; Serafini et al., 1994). By showing that populations of callosal, but not spinal, commissure neurons require GAP-43 to respond to netrin-1, our results therefore provide an explanation for why the GAP-43 ($-/-$) mouse fails to form a corpus callosum *in vivo* whereas the ventral spinal commissure is normal. They also demonstrate that the ability of netrin-1 to guide neurons across the midline depends on how the response of the commissural neuron is exerted intracellularly at the intersection between the membrane and the cytoskeleton, as well as how the guidance cue receptor DCC is activated. The callosal commissures of GAP-43 ($+/ -$) heterozygotes are also defective *in vivo* (Shen et al., 2002). Our results here indicate that this haploinsufficiency is due, at least in part, to failure of the callosal neurites to respond to netrin-1-mediated chemotropic guidance and/or outgrowth. We have also previously shown a strong dose-dependent correlation between the degree of callosal deficit *in vivo* and the level to which GAP-43 is phosphorylated by PKC (Shen et al., 2002), a function known to involve activation of Ig-superfamily extracellular receptors, like DCC (Meiri et al., 1998). Together these results suggest that a functional threshold of GAP-43 is required for netrin-1's ability to stimulate axon crossing across the telencephalic midline.

4.2. Netrin-1 has distinct effects on cortical efferents

Our results also show that GAP-43 dependence can be used to distinguish between distinct netrin-1-mediated effects on different types of cortical efferents that are generated at approximately the same time (Koester and O'Leary, 1993, 1994). These efferents form distinct pathways; ventrolaterally directed subcortical projections are extended first (around E12 in mice) and descend through the internal capsule to synapse subcortically in thalamus, midbrain, hindbrain and spinal cord (Wise and Jones, 1977). In contrast callosal axons are extended much later, at around E15 in mice, (Koester and O'Leary, 1994) and are guided medially across the midline to synapse in the contralateral hemisphere (Czeiger and White, 1993). Both classes of cortical efferents appear to require netrin-1 function (Metin et al., 1997; Richards et al., 1997; Serafini et al., 1996). We showed here that, as in the callosal axons, netrin-1 (whether recombinant or expressed by floorplate) was able to stimulate chemotropic guidance and increase the numbers of ventrolateral axons. In contrast, since the population of maximally responsive neurons appeared to be absent from these explants, netrin-1 affected outgrowth to a lesser extent. Nonetheless, and in contrast to the callosal axons, netrin-1 was able to stimulate chemotropic guidance of ventrolateral axons even if GAP-43 was absent, and only outgrowth and numbers of neurites were GAP-43 dependent.

Not all effects of netrin-1 involve regulating behavior of neurites. Netrin-1 can also have a direct effect on either neuronal survival (Llambi et al., 2001) and on differentiation (Mancino et al., 2009), both mediated *via* DCC. This co-culture model only allows chemotropic guidance responses to be distinguished from outgrowth/survival responses, but not survival to be distinguished from differentiation, hence whether netrin-1 is promoting survival or differentiation of neurons in this instance is not clear and will be addressed in future experiments. We have previously shown that failure to express GAP-43 can decrease neuronal survival and prematurely inhibit differentiation of neuronal precursors *in vivo* (Mani et al., 1999). These results, which show that GAP-43 has no effect on survival/differentiation in the absence of netrin-1, suggest that previous *in vivo* results we reported may also reflect a contribution by netrin-1. This too requires further investigation. The alternative explanation that the increase in neurite numbers seen in the cortical explants merely reflects increased induction of outgrowth appears less plausible given that we are able to clearly distinguish between the ability of netrin-1 to stimulate outgrowth

but not numbers of spinal neurons. Likewise the possibility that the demonstrated effects are due to differential toxicity of the collagen gels seems unlikely given the previously reported anti-apoptotic behavior of collagen in this assay (O'Connor et al., 2001).

4.3. Molecular mechanisms underlying netrin-1 mediated effects

Each of the classes of neuron we studied required functional DCC in order to generate their response to netrin-1, even though their dependency on GAP-43 to execute the response differed. DCC, as well as the other netrin-1 receptors thus far identified, all have structural characteristics of the Ig superfamily of cell adhesion molecules (Ig-SF CAMs, Tessier-Lavigne and Goodman, 1996). This is notable because we have previously shown that GAP-43 is required for neurite outgrowth responses to a number of Ig-SF CAMs, such as NCAM, L1 and N-cadherin, *in vitro* (Meiri et al., 1998). These Ig-SF CAMs are all able to stimulate GAP-43 phosphorylation *via* a pathway that involves activation of PI3, PLC γ and PKC (Gupta et al., 2010; Meiri et al., 1998). PLC γ activation has also been implicated in netrin-1 mediated signaling (Ming et al., 1999). The correlation between levels of GAP-43 phosphorylation in callosal axons and the severity of the *in vivo* phenotype in GAP-43 ($+/ -$) mice (Shen et al., 2002), together with the results here showing that netrin-1 effects on callosal axons are also reduced in GAP-43 ($+/ -$) mice, leads us to propose that netrin-1 dependency of on GAP-43 may also be mediated *via* GAP-43 phosphorylation by PKC. On the other hand GAP-43 expression and phosphorylation remain high in callosal axons *in vivo* even after they have crossed the midline and left the source of netrin-1, suggests that GAP-43 is responsive to other extracellular signals in addition to netrin-1. Likewise the results here showing that not all of netrin-1 effects are abrogated when GAP-43 is absent further confirm that netrin-1 is not linked to GAP-43 in a precisely linear fashion. If these hypotheses are correct we might expect that in instances where netrin-1 effects are not GAP-43 dependent, GAP-43 levels and its phosphorylated form should be low indicating that it has not been activated. In support of this notion, GAP-43 expression in spinal commissure axons *in vivo* is much less than in cortex and is rapidly downregulated after the midline has been crossed (Fitzgerald et al., 1991; Kawasaki et al., 2001). Likewise, GAP-43 in spinal commissures *in vivo* is also not phosphorylated by PKC (Dent and Meiri, 1998).

In growth cones, phosphorylated GAP-43 is localized to areas of expanding lamellae and extending filopodia, and can stabilize actin filaments (Dent and Meiri, 1998; He et al., 1997; Nguyen et al., 2009). Hence, as would be expected if GAP-43 dependent stimulation of chemotropic guidance also requires DCC mediated phosphorylation of GAP-43, GAP-43 ($-/-$) and ($+/ -$) callosal growth cones are significantly smaller and have less F-actin than their wild type counterparts, whereas spinal commissure growth cones are unchanged (Shen et al., 2002).

Our results that netrin-1-mediated attractive guidance of ventrolateral neurites *in vitro* can occur whether or not GAP-43 is present suggests that netrin-1/DCC may work through multiple intracellular signaling pathways to regulate attractive guidance of cortical efferents. Alternatively it has been suggested netrin-1/DCC may not be the only attractive guidance signal directing outgrowth of the ventrolateral neurons (Richards et al., 1997). The ventrolateral phenotype in the netrin-1 hypomorph is much less severe than the callosal phenotype, showing disorganization rather than complete disruption (Braisted et al., 2000). Moreover levels of phosphorylated GAP-43 are higher in ($+/ -$) ventrolateral efferents than in callosal efferents *in vivo* (Shen et al., 2002). Both results are consistent with the notion that GAP-43 phosphorylation can be stimulated by more than one pathway, but do not distinguish whether the divergence occurs extracellularly or intracellularly.

Behavior of the DCC receptor itself may provide a clue as to why some netrin-1 responses require GAP-43 but others do not. DCC functions are mediated subsequent to formation of a complex, and can be modified by changing the DCC binding partners. Hence DCC interaction with unc5 can convert attraction to repulsion (Hong et al., 1999). More relevant to the situation with GAP-43, DCC interaction with ROBO can silence the netrin-1 mediated attraction without converting it to frank repulsion (Stein and Tessier-Lavigne, 2001). Our results showing DCC to be required in all the responses we measured, even those that do not require GAP-43 suggest that the GAP-43 dependence may be mediated via a binding partner rather than DCC itself. Functional interactions between DCC binding partners and GAP-43 need not imply direct interactions between them: in growth cones GAP-43 is located in cholesterol-enriched microdomains together with elements of the NCAM/FGFr signal transduction pathway, and its phosphorylation appears to stabilize these associations although NCAM and GAP-43 do not appear to interact directly (He and Meiri, 2002). Whether GAP-43 behaves similarly to stabilize DCC-mediated signal transduction pathways remains to be seen.

4.4. GAP-43 in chemotropic guidance and neurite outgrowth

This is the first time that neuronal responses to netrin-1 have been directly demonstrated to require GAP-43, although our evidence showing that cortical cytoarchitecture is severely disrupted *in vivo* when GAP-43 is absent has implied this result. Taking previous data into account, we speculate that these results are in fact showing that members of the Ig-SF CAM superfamily, of which the netrin-1 receptor DCC is a member, converge onto a similar repertoire of intracellular cytoskeletal regulatory mechanisms. Even so, the fact that different classes of netrin-1-dependent cortical efferents can be distinguished based on their requirement for GAP-43, despite being generated within the same narrow time frame, further suggests that the assembly of netrin-1 responsive mechanisms within individual neuron types is highly specific and dynamically regulated. Understanding which other extracellular and intracellular components contribute to these mechanisms, and whether netrin-1 effects on survival and differentiation are also involved in these behaviors will be an important direction of future studies.

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